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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The aromatic hydrocarbon (or dioxin) receptor (AhR) which is a ligand-activated basic helix-loop-helix (bHLH) transcription factor, mediates the toxic responses of dioxin. The research in this proposal is testing the hypothesis that the dioxin receptor (AhR) plays a central role in regulating the mammary epithelial network during the gland development, as well as regulating major events in mammary carcinogenesis. Following on preliminary observation of the dramatic upregulation of this receptor in advanced human breast carcinoma (HBC) cell lines, we proposed to address the question of whether the AhR overexpression alone is sufficient for transforming normal mammary epithelia, and whether it is causally associated transformation, using two genetic approaches. The AhR expression will be blocked in high tumorigenic HBC cell lines by siRNA technology to demonstrate a direct role of the AhR in modifying the progression of metastasis. To directly address the effect of increased expression of AhR, the human AhR cDNA will be stably transfected and over-expressed in a normal mammary epithelia and in non-tumorigenic human breast cell line. The development of metastatic phenotypes in the AhR-transformed lines will be assayed as their ability for anchorage-independent growth in soft agar media and for inducing tumors in nude mouse.

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Introduction

The proposed research studies are based on our novel observation that in three different sets of human breast carcinoma (HBC) cell lines the expression of the Aryl hydrocarbon receptor (AhR) protein was up-regulated in a direct correlation with the progression of tumorigenecity. The overall objective is to determine the role of AhR in human breast carcinogenesis. AhR which is well known for its mediation of the toxic responses to environmental polyhalogenated aromatic hydrocarbons (PAH) such as dioxin (1), is a cytosolic protein and binding of PAH leads to its activation to a nuclear transcription factor and subsequent down-regulation by proteolysis (2,3,4). Molecular cloning and characterization of AhR cDNA has identified it as a member of family of ligand-activated basic helix loop helix (bHLH) transcription factors (5). PAH-activated AhR heterodimerizes with its partner AhR nuclear translocating protein (ARNT) another bHLH transcription factor and induces the expression of a number of genes, including cytochrome P4501A1 (CYP1A1) and CYP1B1 (reviewed in 6 & 7). No endogenous ligand for the AhR has yet been identified, however, its constitutive activation via disturbing cellular adhesion to the extra-cellular matrix (8,9), increasing intracellular Ca2+ (10), and disturbing cytoskeleton (11) has provided evidence for physiologically-activated pathways linked to adhesion. Furthermore, other lines of evidence are gathering to implicate the AhR in normal development and tissue homeostasis. For instance, TCDD exposure in animals induces teratogesis, immunosuppression, reproductive defects and tumor promotion, in an AhR-dependent manner. The dioxin-dependent activation of the AhR has also been linked to inhibition of proliferation in mammary and uterine tumor cell lines (reviewed in 12), and enhanced terminal differentiation in keratinocytes and palatal epithelia (13,14). Moreover, the AhR null mice generated by two independent laboratories are normal and fertile, exhibiting a spectrum of hepatic and immune system defects (15,16), but are resistant to benzo(a)pyrene-induced skin and liver carcinogenicity (17). In cultured cells, TCDD shows marked effects on cell cycle progression, where it induced a cell cycle arrest at G1/S check point, an effect that is mediated through the AhR and it involves the induction of cyclin-dependent kinase (CDK) inhibitor p27[kip1]. The AhR in absence of dioxin or other ligands, was shown to influence cell cycle progression, cell shape and differentiation (18, 19). This effect on the cell cycle progression relies on a direct protein-protein interaction of AhR with retinoblastoma (Rb) through an LXCXE domain on the AhR (20). Howeverr, studies in MCF-7 showed that AhR associates with Rb only after receptor activation and nuclear translocation (21). Furthermore, this interaction with Rb protein is required for the maximal AhR transcriptional activity (22). Although TCDD acting through AhR is a potent tumor promoter in mouse skin and in rat liver, it has strong anti-mitogenic effect in estrogen-responsive tissues and exhibits a broad spectrum of anti-estrogenic activities in human breast carcinoma cells (reviewed in 12). The AhR-null mouse or normal mouse treated with TCDD, exhibited impaired development of mammary gland ductal branching (23). Taken together with the anti-mitogenic effect exerted by TCDD on these cells, and the fact that TCDD down-regulates the AhR subsequent to its activation, these findings suggests that AhR is involved in regulating the proliferative stage required for mammary gland development. Preliminary investigations in our laboratory have demonstrated the expression of high levels of AhR protein in human mammary carcinoma cell lines in direct proportion to their degree of tumorigenicity and metastatic potential (24). We hypothesize that the AhR plays a major role in regulating mammary epithelial network during mammary gland development, and its over-expression contributes to the development of metastatic phenotypes in human breast carcinoma. The initial aim of this work has been to investigate the mechanisms of AhR involvement in regulating mammary epithelia both during development and tumoigenesis.

BODY

Much progress has been made during this year. We have exceeded some of our planned tasks, however we had a modest progress as a far as the animal studies. Mainly we were hampered by extremely slow

breeding of the AhR knock-out (AhR-KO) mice. Overall, we are on target and we are anticipating this following year will be intense since it will be the harvest year.

Objective 1: Restoration of the normal mammary development in the AhR-KO mice by transfecting AhR cDNA into their mammary tissues in situ using retroviral expression vector.

Task1: the retroviral expression vectors for both mouse and human cDNA were successfully cloned and high titer viruses were produced from each and stored for these experiments and other projects. The titer for the mouse AhR viruses was determined in mouse embryo fibroblasts (MEF), which we generated from AhR-KO embryos. The titer was determined both based on GFP and AhR protein expression (Appendix Fig. 1).

Task 2. Breeding and generation of the AhR-KO mice colonies: this proved to be one of our most difficult tasks. We initially managed to accumulate about 14 AhR-/- breeding pairs, however they were not fertile and the few that got pups, they were either too weak to reach the term of the experiment. So we recently changed our breeding regimen, where we bred AhR+/- and AhR-/- and genotyped the offsprings for the AhR-KO females for the experiments. So far it seems that we are successful in getting some animals.

Task 3: Pilot experiments with retroviral vectors carrying a marker gene to optimize injections and expression efficincy. This tasks was completed successfully, mainly because we are using the GFP-retrovectors, which allowed us to use the heterozygous pups for the optimization experiments. We found out that we have to modify some of procedures that we initially proposed. For instance, we proposed to infuse the viruses via the mammary teats (using their nipples as guide). This procedure was practically impossible, at the four-weeks (when we start the infection), the mammary glands are very rudimentary and could hardly be seen. So we modified the procedure to inject the viral suspension in the whole gland area (including both the stromal fat pad and the glandular tissues). By this procedure we were successful in delivering the viruses and expression of both GFP and AhR in the whole gland area (Appendix Figure 2).

Task 4: Processing of mammary glands collected from above experiments. We successfully finished most of this task. We used the help of the laboratory personnel of Dr. Hal Moses at Vanderbilt Cancer Center in establishing our expertise in collecting the mouse mammary glands and processing the whole gland mounts. Following the capturing of the images of the whole gland branching ducts development, we are sending the glands to Vanderbilt Histopathology services, where they do the paraffin mounting and sectioning. We receive the slides with multiple sections for in situ hybridizaion, immunohistochemical staining and fluoroscopic examination. We have developed an in situ hybridization assay for AhR (probes are tested for both human and mouse AhR) (Appendix Figure 3).

Task 5: Preparation of whole gland extracts and Western blotting analyses for AhR etc. The methodology for this is done, where we were successful in flash-freezing the dissected glands from different areas of the mouse (Left Vs right inguinal, abdominal and thoracic mammary glands), then pulverizing the frozen glands and solublizing the powder in Trizol reagent, which allowed us to isolate RNA, DNA, and proteins, successively.

Task 6: Collection of data, analysis and manuscript preparation and submission: This task is in progress, since we collect data as we go along. Some manuscripts are in preparation.

Objective 2: To determine the status of the AhR activation in the Sager's cells in presence or absence of TCDD treatment.

Task 7: Perform activation experiments with Sager's cells. These experiments are done and data were analyzed and a manuscript is in preparation to be submitted by this Fall.

Task 8: Run electromobility shift and collect data..This has not been done yet

Task 9: immuno-chemical staining and fluorescence localization of AhR in Sager's cells..Done to be part of the manuscript in Task 7.

Objective 3: Over-expression of AhR in non-tumorigenic human breast carcinoma cell

Task 10: Months 25-27: Generation of stable cell lines from Sager's 21PT HBC and normal mammary epithial cell lines, by stable transfection of huAhR cDNA using retroviral transfection system. This task is underway, where we managed to infect the H16N2 normal immortalized mammary epithelial cells with human AhR-expressing viruses. We are in the process of optimizing the infection and selecting cell clones with high AhR expression. (Appendix Figure 4)

Task 11: Months 28-29: Characterization of the AhR-transformed 21PT and H16N2 cell lines for their proliferative (cell cycle analysis), apoptotic capacity and expression of metastatic determinants. Preliminary characterization is initiated (Appendix Fig 5).

Objective 4: Blocking of AhR expression in highly metastatic Sager's 21MT2 human breast carcinoma cell line by stably transfecting antisense human AhR cDNA.

Task 12: Months 25-27: Transfection and generation of AhR antisense cDNA-transformed MT cell lines. (This will be done simultaneously with Task 10).

Antisense has been replaced with small interference RNA (siRNA) technologies, as explained in last year report. We have developed four siRNAs and packaged them in recombinant plasmids, which were designed to produce hairpin RNAs targeted against AhR These recombinant plasmids are currently being used to transfect the highly metastatic cell line (21MT2) to suppress the AhR expression in these cells. While we are fine-tuning this transfection efficiencies and expression levels, we are setting-up and validating the invasion assay for screening these cells. We are suppressing the AhR expression in these cells with TCDD, since TCDD treatment causes down-regulation of the AhR, this strategy was used to chemically test the hypothesis that decreased AhR expression will lead to decreased invasive properties of this cell line. MT2 cells were treated with 1nm TCDD for 24 hours. Treated and untreated cells were evaluated for their invasive potential using the FluroBlok invasion system.

Task 13: Months 28-29: Characterization of the AhR antisense cDNA-transformed 21MT cell lines for their proliferative (cell cycle analysis), and apoptotic capacity and expression of metastatic determinants. (This will be simultaneously with Task 11). Progress is same as in Task 11.

Objective 5: Characterization of transformed cell lines generated in Objectives 3 and 4, for their tumorigenic and invasive phenotypes.

Task 14: Months 28-33: Mice inoculation with transformed cell lines and monitoring of mammary tumor formation. Not initiated yet.

Task 15: Months 29-31: Measurement of anchorage-independent growth on soft agar media. Not initiated yet.

Task 16: Months 32-35: Measurement of invasiveness using in Fluorescence-based invasion assay. Task is initiated and the assay is being validated.

Task 17: Months 28-36: Analyses of the collected data and preparation of the results for publication. In progress.

Key Research Accomplishments

- Development of retroviral expression vectors and production of retroviruses expressing genes for both human and mouse AhR.
- Development and banking of Phoenix packaging cells stably producing high titer AhR viruses
- Demonstration of the capability of these viruses to stably express high levels of both AhR gene and GFP marker gene in cultured cells and in mice mammary glands.
- Successfully establishing for the first time, a method for in situ viral infection to mouse mammary glands, as a means of introducing genes in a mammary gland with a negative background for the specific gene.
- Design and synthesis of four siRNA for human AhR
- Development and banking of AhR-KO mouse embryo fibroblast, for use as screening virus titer for AhR.
- Demonstration of constitutive activation of AhR in the advanced breast carcinoma cells lines and its association with Cytokeratin-18 (in preparation for submission for publication).

Reportable Outcomes

- Abstract and a poster were presented at the Society of Toxicology meeting 2002.
- Manuscript is in preparation to be submitted by this Fall.
- Preliminary data from this grant is being used to prepare an RO1 research proposal to be submitted to the NIEHS.

Conclusions

We have observed, in support of our working hypothesis, that the level of expression of the AhR have a great impact on the proliferation of the mammary epithelia (both from the mice mammary glands experiments and the H16 human mammary epithelial cells. This observation and the modification of invasiveness of the MT2 cells by TCDD treatment (which chemically knocks down the AhR expression) are remarkable findings which indicate that this receptor might hold the key for link between the development of the mammary gland and the progression of breast cancer.

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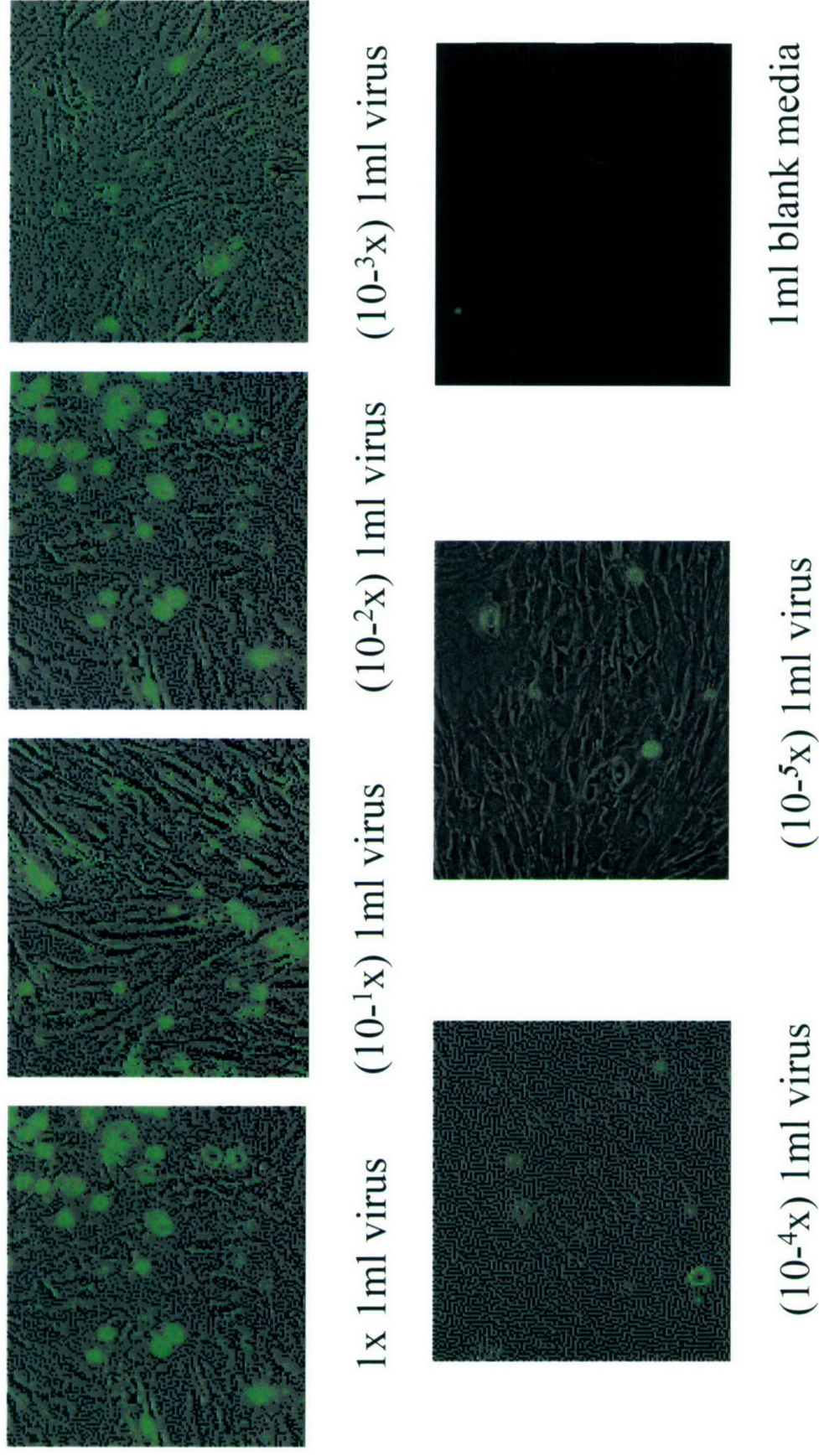
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Appendix 1

1x 1ml virus   (10⁻¹x) 1ml virus   (10⁻²x) 1ml virus   (10⁻³x) 1ml virus

(10⁻⁴x) 1ml virus   (10⁻⁵x) 1ml virus   1ml blank media

Figure 1A. the AhR-GFP virus expression titer in AhR⁻/⁻ mouse embryo fibroblasts

Appendix 2

Human AhR (104 kDa)

Mouse AhR (95 kDa)

9   8   7   6   5   4   3   2   1
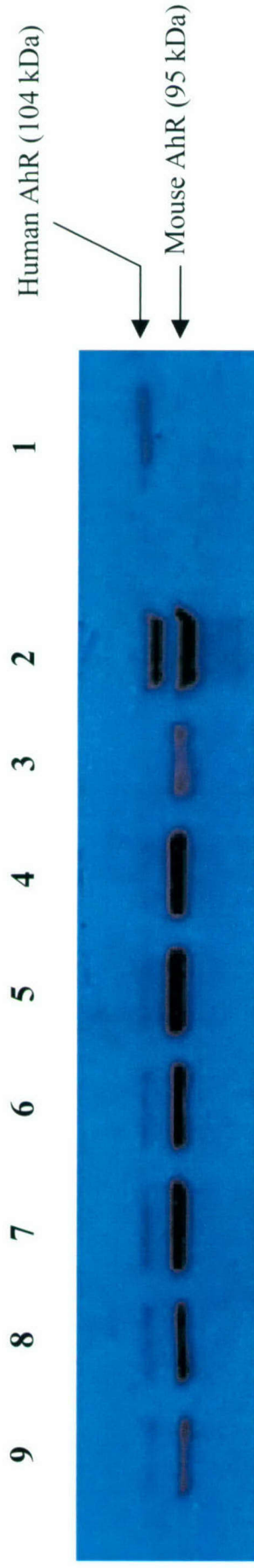
Figure 1 B. Western blot showing the expression of AhR protein from the GFP-retroviral vector containing the cDNA of mouse AhR. Twenty microgram of 2%SDS cellular lysates of the respective cells was electrophoresed on 7.5% PAGE and electro-transferred to PVDF membrane. The membrane was probed with AhR-specific rabbit polyclonal antibodies, followed by HRP-conjugated goat anti-rabbit and the specific AhR-reactive bands were visualized by chemiluminscence detection method.

Lane **1**: Phoenix packaging cells alone (untransfected)
Lane **2**: Phoenix cells transfected with retroviral vector of mouse AhR cDNA (5days-post transfection)
Lane **3**: mouse embryo fibroblasts from AhR+/- mouse
Lanes **4&5**: Phoenix cells transfected with retroviral vector containing mouse AhR cDNA at pH 6.9
Lanes **6&7**: Phoenix cells transfected with retroviral vector containing mouse AhR cDNA at pH 7.0
Lanes **8&9**: Phoenix cells transfected with retroviral vector containing mouse AhR cDNA at pH 7.1

Results showed that the phoenix cells express endogenous AhR that has the typical size of the human AhR (104 kDa). The mouse cDNA, as expected is expressed as 95 kDA (B-1 type), which was identical to the AhR endogenously expressed by the c57Bl/6 mouse which is the background mouse for the knock-out mice. These results and others established that we get the optimal expression (as well as highest virus titer) when calcium phosphate transfection of retrovector is done at ph 6.9.

Appendix 3

Human AhR (104 kDa)
Mouse AhR (95 kDa)

Mouse Embryo Fibroblasts

H16 (no virus)

H16 + control vector virus

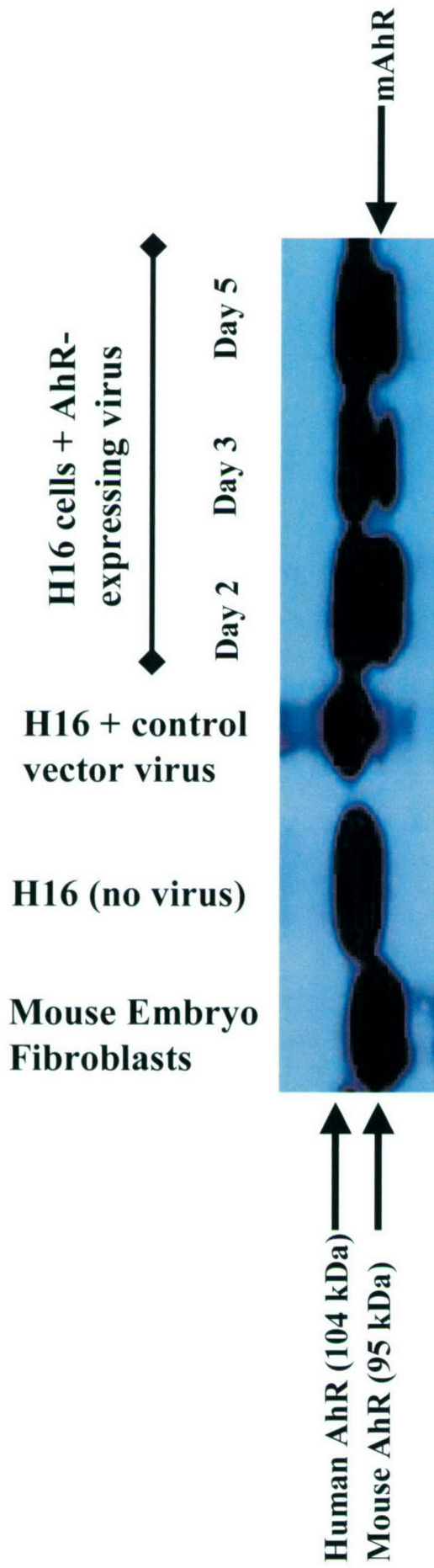H16 cells + AhR-expressing virus

Day 2   Day 3   Day 5

mAhR

Figure 1C. Testing of the titer of viral stocks produced by trasfecting phoenix packaging cells with pBMN-GFP retrovector control or carrying mouse AhR cDNA. Viruses wrer collected from packaging cells when they were almost completely transfected as evident by the green fluorescence from GFP expression (Figure 3). Media containing viruses are collected 48 hours, 3 days or 5 days post transfection. I:1 of the virus to media is added to test cells (immortalized human mammary epithelial cells H16N2). Cells are collected 24 hour after infection, lyzed in 2% SDS lysis buffer, and 20 mg were analyzed by Western blotting for AhR. Results confirmed that these viruses are capable of infecting these mammary cells and expressing the mouse AhR protein to a substantial level.

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Figure 1 D. Phoenix cells transfected by calcium phosphate method at pH 6.9 with pBMN-GFP retrovector containing the mouse AhR cDNA. Fluorescence images of the phoenix cells were taken 48 h post transfection, at the time of virus collection.
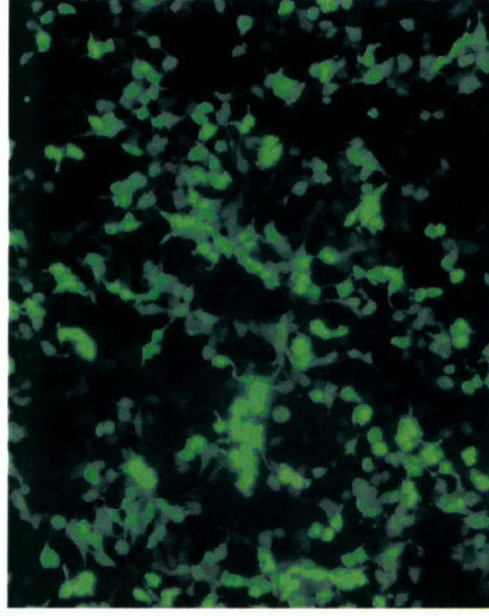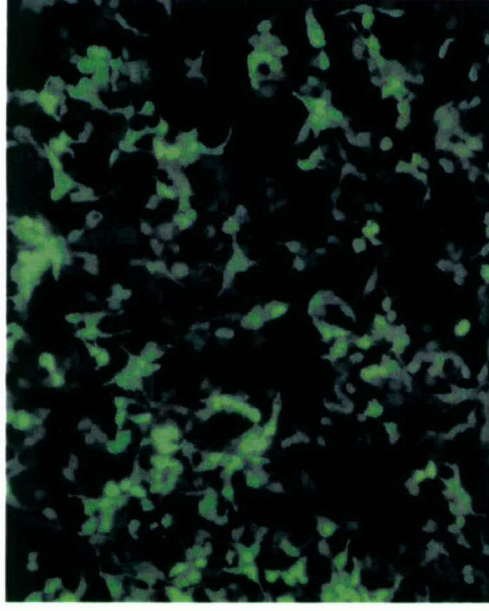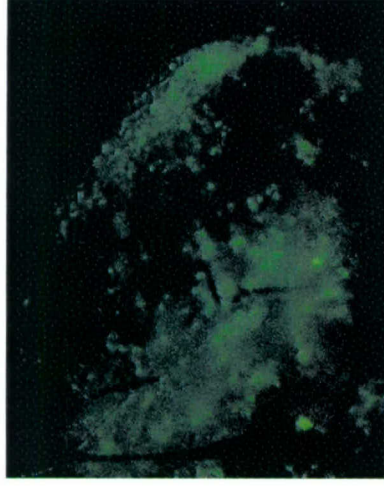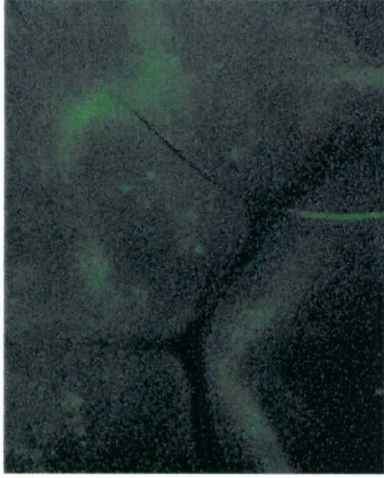
H16N2 the immortalized human mammary epithelial cells were infected with virus supernatant from phoenix cells above, collected 48 h after transfection. GFP fluorescence images of H16 cells were captured 24h after viral infection.
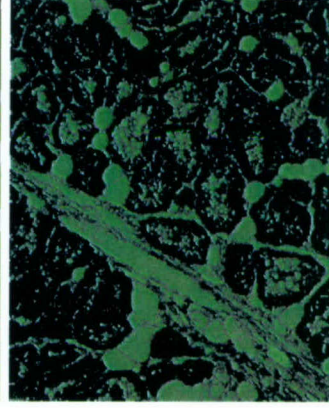
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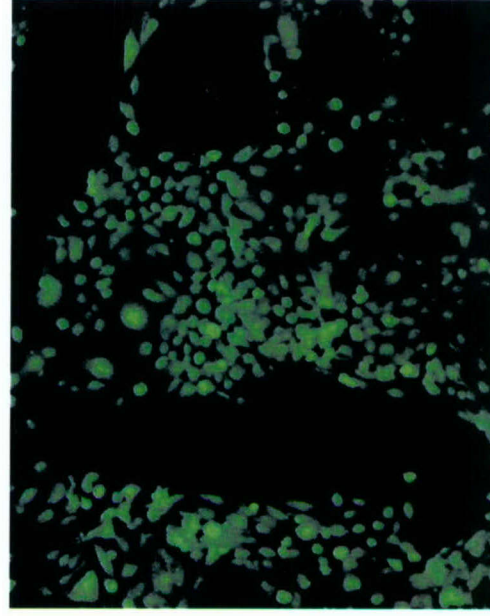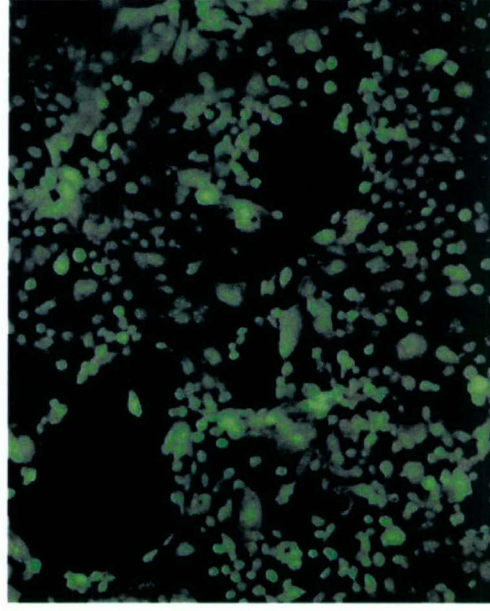
Whole
Gland

In histological
sections

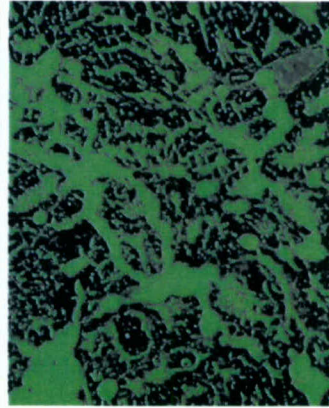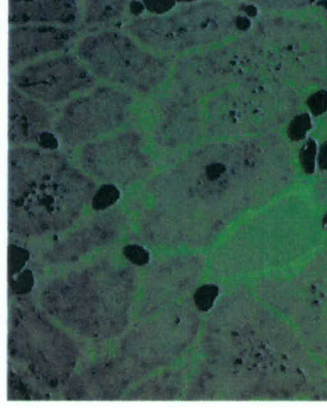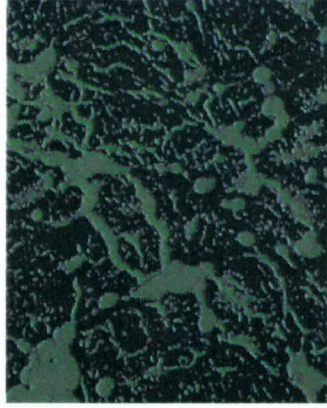Figure 2. expression of GFP-carrying retro-vectors in mammary glands of 8 week-old AhRKO mice
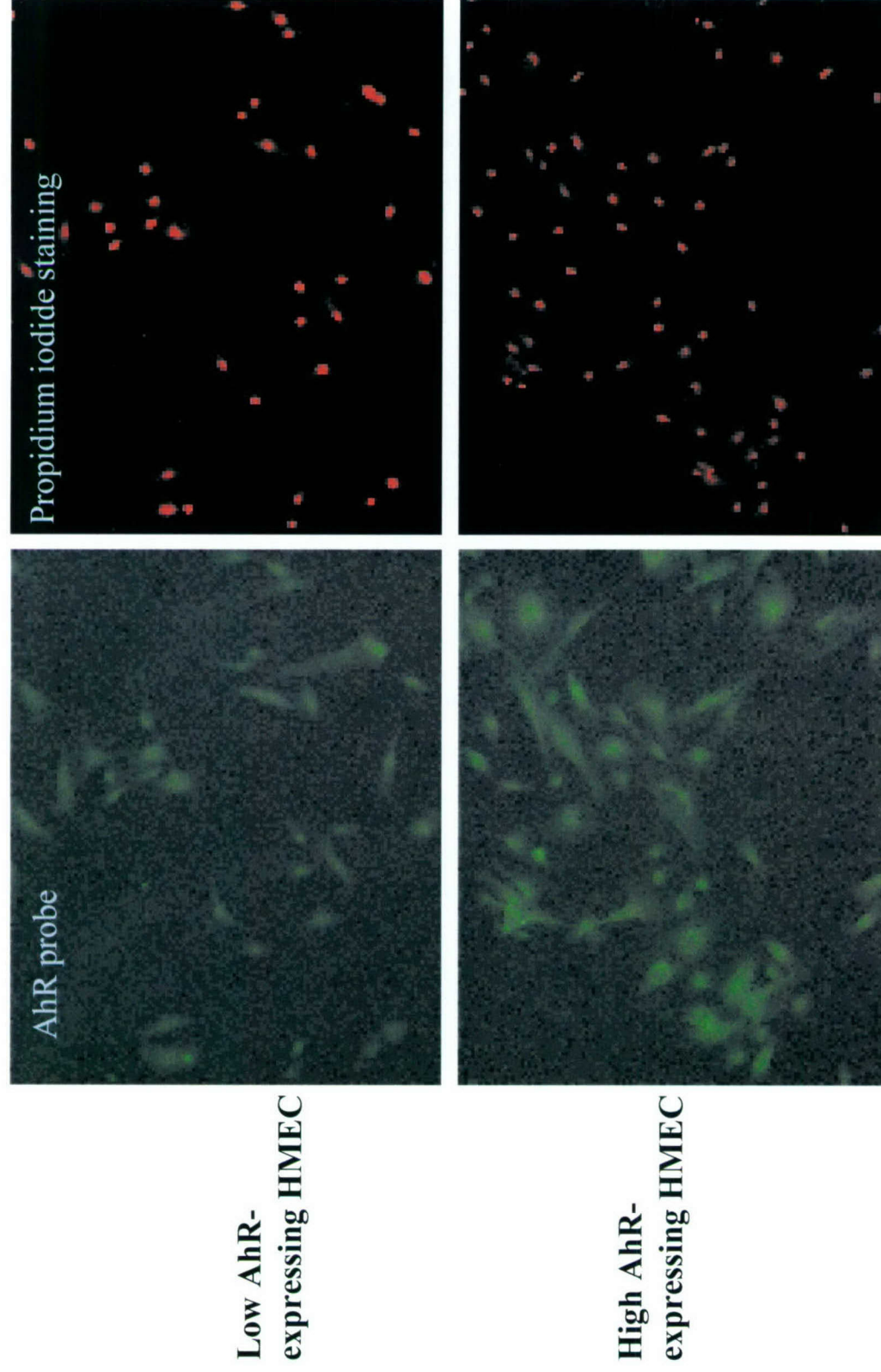
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Appendix 6

Figure 3. AhR expression in primary human mammary epithelial cells (HMEC), by In situ hybridization. The fluorescence riboprobes were synthesized and used to probe for the AhR RNA in HMEC grown on glass cover-slips and fixed with methanol/acetone. The hybridization method was developed in the laboratory using a commercially available kit and aided by the providor's instructions.
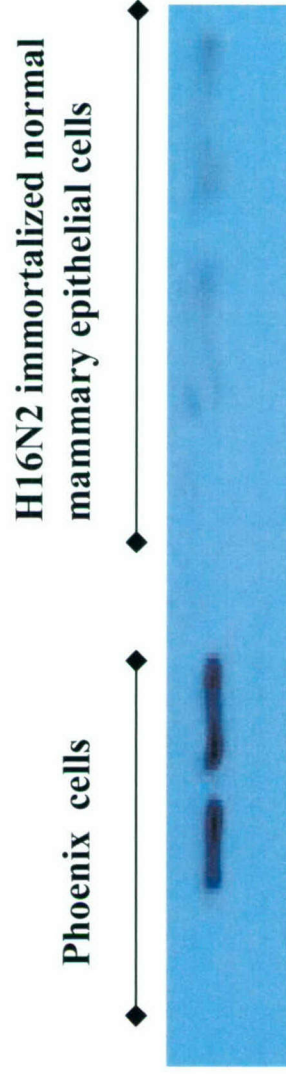
15

Appendix 7

The final recombinant retrovirus vector containing AhR cDNA mixed with GFP vector was used to transfect Phenoix packaging cell line

Phoenix cells

H16N2 immortalized normal mammary epithelial cells

retroviral particles were collected from phoenix cells media and were used to infect immortalized normal mammary epithelial cells (H16N2). The positive cells were selected by antibiotic selection with G418

Phoenix cells

H16N2 immortalized normal mammary epithelial cells

Figure 4. AhR expression in the infected and non-infected H16N2 cell lines was determined by western blotting.
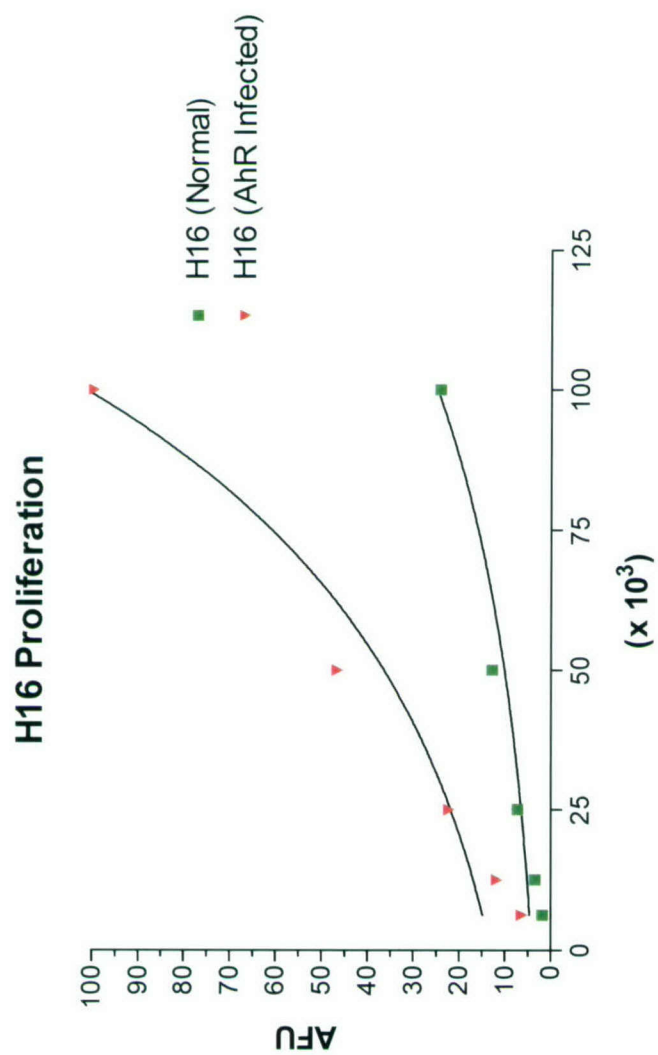
Appendix 8

H16 Proliferation

Figure 5: Proliferation of H16 infected with retrovirus to over-express AhR-expressing H16N2 increased in proliferation by 4-fold